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published in
The Plant Cell
2000

DOI (link to publisher)
10.1105/tpc.12.9.1619

document version
Publisher's PDF, also known as Version of record

The Plant Cell, Vol. 12, 1619–1631, September 2000, www.plantcell.org © 2000 American Society of Plant Physiologists

anthocyanin1 of Petunia Encodes a Basic Helix-Loop-Helix Protein That Directly Activates Transcription of Structural Anthocyanin Genes

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The petunia loci *anthocyanin1* (*an1*), *an2*, *an4*, and *an11* are required for the transcription of anthocyanin biosynthetic genes in floral organs. The *an2* and *an11* loci were recently cloned and shown to encode a MYB-domain transcriptional activator and a cytosolic WD40 protein, respectively. Here, we report the isolation of *an1* by transposon tagging. *an1* encodes a new member of the basic helix-loop-helix family of transcription factors that is functionally and evolutionarily distinct from JAF13, the apparent petunia ortholog of maize RED1 and snapdragon DELILA. We provide genetic evidence that the transcription factors encoded by *an1*, *an2*, and *an4* operate in an unexpectedly complex regulatory hierarchy. In leaves, ectopic expression of AN2 induces *an1* expression, whereas in anthers, *an1* expression depends on *an4*, encoding (or controlling) a MYB protein that is paralogous to AN2. Experiments with transgenic plants expressing a post-translationally controlled AN1–GLUCOCORTICOID RECEPTOR fusion protein indicated that independent of protein synthesis, AN1 directly activates the expression of the *dfrA* gene encoding the enzyme dihydroflavonol 4-reductase and of *Pmyb27* encoding a MYB-domain protein of unknown function.

INTRODUCTION

Pigmentation is a phenotypic trait that provides an excellent system for studying how groups of genes are coordinately expressed in a tissue-specific manner (reviewed in Mol et al., 1995, 1998). Synthesis of anthocyanin pigments from malonyl-CoA and 4-coumaroyl CoA requires some 10 to 15 enzymes, and many of the structural genes encoding these enzymes have been isolated (Holton and Cornish, 1995). Regulatory genes that control the tissue-specific expression of structural anthocyanin genes have been identified by mutation in several species.

In maize, a MYB domain and a basic helix-loop-helix (bHLH)-type transcription factor, encoded by the *colorless1* (*c1*)/*purple leaf1* (*pl1*) and the *red1* (*r1*)/*booster1* (*b1*) gene family, respectively, are required for activation of structural anthocyanin genes (reviewed in Mol et al., 1998; Weisshaar and Jenkins, 1998). At least one other factor, encoded by *pale aleurone color1* (*pac1*), is required for activating the structural genes in the maize kernel (Selinger and Chandler, 1999). The nature of the PAC1 protein, however, is not known. In two-hybrid assays in yeast and maize cells, B1 interacts with C1, which suggests that both proteins are in the same transcription complex (Goff et al., 1992). In vitro, C1 binds with low affinity to functional *cis*-acting elements in the promoters of structural genes (Sainz et al., 1997), but thus far, no DNA binding activity has been detected for R1. Whether this is because R1 needs to dimerize with an as yet unknown partner or because the C1/R1 complex interacts in vivo with the promoter of an unknown "intermediate" regulator is unclear at this stage. The gene *intensifier1* (*in1*) is an inhibitor of anthocyanin synthesis because loss-of-function mutations in this gene result in a more intense pigmentation of the kernel. A small fraction of the *in1* transcripts encode a bHLH protein homologous to R1. However, the large majority of transcripts are misspliced and encode truncated proteins, which may be responsible for the inhibitory character of this locus (Burr et al., 1996).

In flowers and seeds of dicotyledonous species, expression of the structural genes encoding the "early" enzymes of the pathway, which are required for the synthesis of all flavonoid classes, and those encoding the "late" anthocyanin-specific enzymes is controlled by distinct genes (Martin et al., 1991; Quattrocchio et al., 1993, 1998; Shirley, 1996). For example, in petunia flowers, the loci *anthocyanin1* (*an1*), *an2*, and *an11* are required for transcription of anthocyanin-specific genes such as *dfrA* (encoding dihydroflavonol 4-reductase; Huits et al., 1994a), *rt* (encoding anthocyanin rhamnosyl-transferase; Kroon et al., 1994), and *an9* (encoding a glutathione *S*-transferase; Alfenito et al., 1998) but not for expression of *chsA* (encoding chalcone synthase), *chi*

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(encoding chalcone-flavanone isomerase), or *an3* (encoding flavanone 3-β-hydroxylase) (Quattrocchio et al., 1993).

The *an2* locus encodes a MYB-domain protein that is functionally interchangeable with C1 from maize (Quattrocchio et al., 1998, 1999). *jaf13* of petunia and *delila* from snapdragon encode highly similar bHLH proteins thought to be orthologs of R1 from maize (Goodrich et al., 1992; Quattrocchio et al., 1998). Ectopic JAF13 expression induces anthocyanin accumulation and transcription of the *dfrA* gene; however, no loss-of-function mutants are known for *jaf13*. The *an11* locus encodes a highly conserved WD40 repeat protein that is localized in the cytosol. Because *an11*$^{-}$ mutants can be rescued, at least partially, by overexpression of AN2, we previously suggested that AN11 may post-translationally regulate the activity of the anthocyanin transcription factors by an as yet unknown mechanism (de Vetten et al., 1997). The observation that mutation of the Arabidopsis *transparent testa glabra* (*ttg*) gene, encoding a homologous WD40 protein (Walker et al., 1999), can be complemented by (over)expression of R1 from maize points in the same direction (Lloyd et al., 1992). The function of *an1* in this regulatory system remains, thus far, unclear because of the elusive nature of the gene product. Recently, Kubo et al. (1999) showed that *anthocyaninless2* (*a2*) of Arabidopsis encodes a protein with similarity to homeodomain transcription factors. However, whether ANL2 coregulates the anthocyanin-specific genes together with the mentioned MYB, bHLH, and WD40 proteins or is an activator of the early structural genes is unclear.

In this article, we report the isolation and molecular characterization of the *an1* locus. *an1* encodes a bHLH protein that is most similar to IN, and less to R1 and JAF13. Analysis of *an1* expression in *an4*$^{-}$ mutants and in transgenic plants ectopically expressing AN2 shows that AN1 operates downstream of AN2 and AN4. Induction experiments in transgenic plants in which AN1 was placed under post-translational control showed that AN1 directly activated transcription of a structural anthocyanin gene, independent of protein synthesis. These data indicate that anthocyanin transcription factors operate in a regulatory hierarchy.

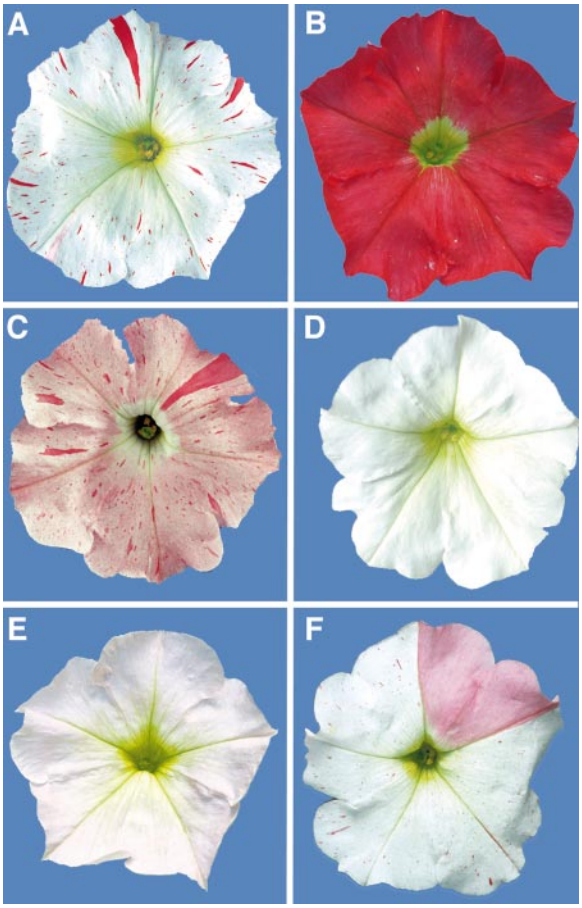

Figure 1. Phenotypes of Flowers Harboring Different *an1* Alleles.

(A) Flower of line W138, homozygous for the allele *an1-W138*.
(B) Flower of a plant heterozygous for *an1-W138* and a full revertant *An1*$^{+}$ allele.
(C) Flower of a plant heterozygous for *an1-W138* (evident from the red spots) and an excision allele with partial activity (evident from the pink background color).
(D) Flower of a plant homozygous for a stable recessive null allele derived from *an1-W138*.
(E) Flower of a homozygote for *an1-A2255*, a stable allele with low activity.
(F) Flower of a homozygote for the unstable *an1-X2191* allele.

RESULTS

Isolation of the *an1* Gene

The petunia line W138 contains an *an1* allele, initially designated *an1*$^{s/+-p}$ but later renamed *an1-W138*, that is somatically and germinally unstable (Doodeman et al., 1984; Huits et al., 1994b). Consequently, *an1-W138* flowers are white with red (full revertant) and pink (partial revertant) spots (Figure 1A). In germinal cells, instability of *an1-W138* results in a fraction of the progeny having completely red, pink, or white flowers (Figures 1B to 1D).

Because somatic instability of the *an1-W138* allele responded to the same genetic element (*Act1*) that is required for transposition of *dTph1* elements (Huits et al., 1994b), we anticipated that *an1-W138* harbored a *dTph1* insertion. To isolate a fragment of the *an1* locus, we cloned *dTph1* flanking sequences unique for *an1-W138* plants by a combination of inverse polymerase chain reaction (PCR) and differential screening of amplification products (Souer et al., 1995). This yielded four genomic fragments that contained a *dTph1* insertion in six different *an1-W138* plants but not in plants homozygous for two independent *An1*$^{+}$ reversion al-

leles. DNA gel blot analysis of a larger set of plants showed that fragment 65 hybridized to a 0.9-kb RsaI fragment in all *an1-W138* homozygotes and to a 0.6-kb fragment in all homozygotes for revertant or stable recessive alleles tested, whereas heterozygotes for *an1-W138* and a revertant allele contained both fragments (data not shown). Because these results indicated that fragment 65 originated from the *an1* locus, we isolated the gene and corresponding petal cDNAs from libraries made from an *An1*$^+$ line. Comparison of the genomic sequence with the cDNA and the original *dTph1* flanking fragment showed that it contained a gene composed of eight exons, which in petunia line W138 contained a *dTph1* insertion at the border of intron 6 and exon 7 (Figure 2A).

Subsequently, we amplified the region containing the *dTph1* insertion from a large number of plants harboring *an1-W138* or derived stable alleles with full (revertant) or null activity. Figure 2B shows that the presence of the *an1-W138* allele resulted in the amplification of a 0.87-kb fragment, consistent with the presence of a *dTph1* insertion, whereas all of the 17 derived stable alleles yielded a 0.58-bp fragment, indicating that these alleles resulted from excisions of that *dTph1* element.

Sequencing of PCR amplification products of *an1-W138* showed that a 284-bp *dTph1* element had inserted at the border of intron 6 and exon 7 and duplicated 8 bp of the target sequence, including the splice acceptor site, whereas the derived stable alleles contained different transposon footprints at this position (Figure 2C). In the full-revertant *An1*$^+$ excision alleles, the footprint sequence contained only one acceptor splice site that fit the consensus sequence (Brown et al., 1996), indicating that exons 6 and 7 were correctly spliced in all transcripts. The two intermediate alleles contain two potential acceptor splice sites. Splicing to the downstream site would produce a functional transcript with a wild-type sequence, whereas splicing on the upstream site would generate a transcript with a frameshift. Sequence analysis of *an1* fragments that were amplified by reverse transcription (RT)–PCR from RNA of *X2198* flowers showed that in vivo, both splice sites indeed were used (data not shown), thus explaining the intermediate phenotype. The stable null alleles *X2198* and *X2273* contain three potential splice sites. Splicing to the most downstream of these would produce a functional transcript, but splicing to either of the two upstream sites would produce transcripts with a frameshift. The stable an1$^-$ phenotypes specified by these alleles suggest that the majority of transcripts are (mis)spliced to one or both of the two upstream sites. In a third null allele, *an1-Z2157*, 195 bp of the right *dTph1* end and 10 bp of flanking sequence, including the splice site, were deleted and only 88 bp from the left end of *dTph1* remained.

One rare excision event produced an *an1* allele, *an1-A2255*, that specifies flowers with a very pale rather than completely white color, indicating that anthocyanin synthesis has been strongly reduced but not completely blocked (Figure 1E). In *an1-A2255*, 248 bp at the left end of *dTph1* and 10 bp of the *an1* flanking (intron) sequence were lost, whereas 36 bp of the right *dTph1* end remained present (Figure 2C); as a result, exon 6 was spliced inframe to a cryptic splice site located ~200 bp downstream in the middle of exon 7 (C. Spelt, F. Quattrocchio, J.N.M. Mol, and R. Koes, unpublished data).

Among W138 progeny, a new unstable *an1* allele was found that specified white flowers in which the revertant spots were predominantly pink and only occasionally red (Figure 1F). Sequencing showed that the *dTph1* element remained in its original position but now flanked by a footprint sequence on its left side (Figure 2C). Presumably, *an1-X2191* arose by a faulty transposition attempt in which only the left end of *dTph1* was cleaved and was repaired again without excision of the transposon, similar to the *an11-G5543* allele (de Vetten et al., 1997).

These results show that genetic alterations of *an1-W138* correlate without exception with (attempted) excisions of the *dTph1* element. Therefore, we conclude that the isolated DNA is derived from *an1.*

an1 Encodes a Novel bHLH Protein

RNA gel blot and RT-PCR experiments showed that the *An1*$^+$ line R27 expresses a major *an1* transcript mRNA of ~2.5 kb. To determine the nature of the AN1 protein, we sequenced two independently isolated cDNA clones. The longest cDNA measured 2454 bp and contained a 2004-bp open reading frame specifying a 668–amino acid protein (Figure 3A). The second cDNA represented a partial cDNA derived from an incompletely spliced *an1* transcript because its 5′ end started 1023 bp downstream from the translation start codon and because it still contained the 473-bp intron located between exons 7 and 8 (data not shown).

Database searches showed that the predicted AN1 protein had structural similarities with several plant bHLH proteins, including those encoded by the *r1* family (R-S, LC1, SN1, and B1) and the *in1* locus of maize, *delila* of snapdragon, and *jaf13* of petunia. Figure 3A shows that AN1 shares a highly conserved N-terminal domain of ~170 amino acids with the other bHLH proteins. The bHLH domain of AN1, located between positions 474 and 522, is virtually identical to that of IN, with somewhat less similarity to the bHLH domains of DELILA, LC1, and JAF13. The C-terminal region downstream of the bHLH domain is in general less conserved between these proteins. In this region, again AN1 shares the most similarity with IN. The middle domain of the bHLH proteins is the least conserved; the JAF13–DEL pair displays the strongest conservation in this region.

To visualize the quantitative similarity between bHLH proteins implicated in anthocyanin synthesis, we constructed the phylogenetic tree shown in Figure 3B, which indicates that these bHLH proteins fall into different classes. One class includes JAF13, DELILA, GMYC, and MYC1, all of dicot origin, and also R_A and the R1 family proteins LC1 and

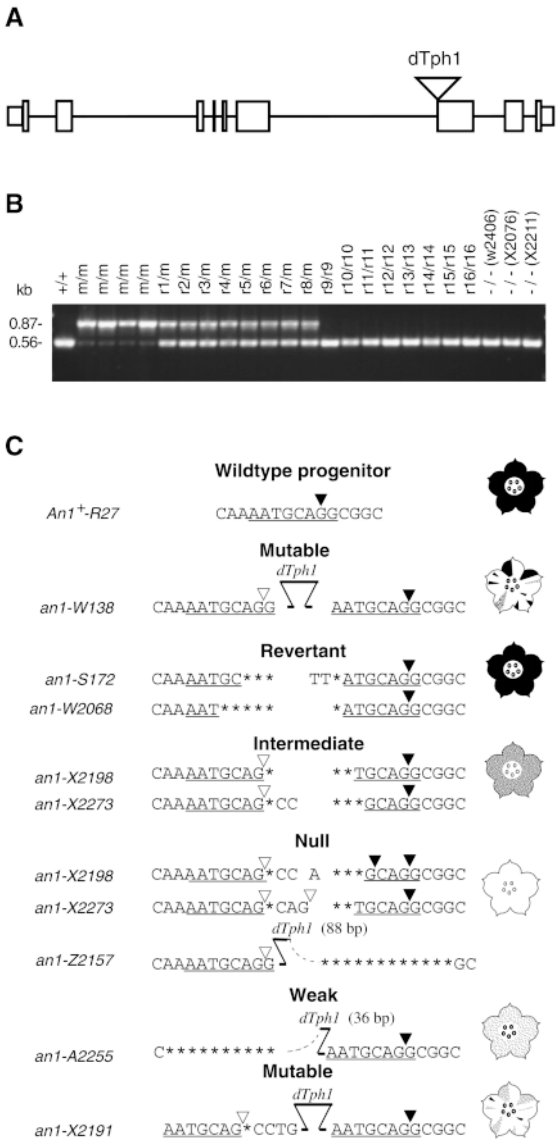

Figure 2. Isolation of the *an1* Gene.

(A) Map of the *an1* gene. Boxes represent exon sequences, separated by thin lines representing introns. Protein coding sequences are indicated by boxes of double height. The *dTph1* insertion in the *an1-W138* allele is represented by a triangle.

(B) Ethidium bromide–stained PCR products amplified from plants harboring the parental *An1-R27* allele (+), the mutable *an1-W138* allele (m), 16 independently isolated full-revertant alleles (r1 to r16), or three independently isolated stable recessive (−) *an1* alleles (W2406, X2076, and X2211). The genotypes indicated above the lanes were determined by phenotypic analysis of progeny obtained by self-pollination. The size of the PCR products is indicated at left.

(C) Sequences of the parental *An1-R27* allele, the unstable *an1-*W138 allele, and derived excision alleles. The flower diagrams indicate the petal color specified by each group of alleles. The 8-bp target site duplication caused by the *dTph1* insertion in *an1-W138* is underlined. Nucleotides that were lost during the attempted *dTph1* excision are indicated by asterisks. The open and closed triangles in *An1-R27* and *an1-X2198* indicate confirmed acceptor splice sites. Triangles in other alleles indicate putative acceptor splice sites that were inferred by using a consensus sequence (Brown et al., 1996). Closed triangles indicate (putative) splice sites that generate an *an1* transcript with an intact reading frame, whereas open triangles indicate (putative) splice sites that generate *an1* transcripts with a frameshift.

B1, all of monocot origin; AN1 and IN, however, seem to represent at least one and possibly two separate classes of proteins. This classification of bHLH proteins is supported by the intron–exon structures of the corresponding genes, as shown in Figure 3C. The protein coding sequences of *b1*, *lc1*, *jaf13*, and r_a contain seven introns in conserved positions (Ludwig et al., 1989; Hu et al., 1996; Quattrocchio et al., 1998), whereas *in1* contains an additional intron that splits the region encoding the weakly conserved middle domain (Burr et al., 1996). This extra intron seems to be conserved in *an1*, although the low sequence similarity in this region makes it impossible to assess whether both introns are in precisely the same position. In addition, *an1* lacks the intron that splits the region encoding the bHLH domain but has a unique intron slightly downstream.

Together, these data show that *an1* encodes a bHLH protein that is not orthologous to R1, DEL, or JAF13, implying that AN1 is a novel regulator of the anthocyanin pathway.

Spatiotemporal Control of *an1* Expression

To analyze the expression pattern of the *an1* gene, we compared the amount of *an1*, *dfr* (an *an1*-controlled structural anthocyanin gene encoding dihydroflavonol 4-reductase), *an2*, *jaf13*, and *gapdh* (encoding the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase) mRNA in various tissues. Figure 4 shows that the limb and tube of developing flower corollas contain relatively large amounts of *an1* and *dfr* mRNA, reaching a maximum around stage 4, when the corolla starts to unfold. *an1* and *dfr* mRNA are also clearly detectable in the pistil, consistent with the presence of anthocyanins in the style. In the petunia line that we used (V30), green tissues such as stem, leaf, and sepal can become partially pigmented, especially in older plants, which coincides with the presence of low amounts of *an1* and *dfr* transcripts. The ovary also contains *an1* mRNA, consistent with the observation that ovaries express *dfr* in an *an1*-dependent manner, even though they do not contain anthocyanins (Huits et al., 1994a). We did not, however, detect *an1* transcripts in root, a tissue that never expresses structural anthocyanin genes.

Expression of *an2* is essentially limited to the limb, with somewhat less expression in the tube and the pistil, which is consistent with the finding that mutation of *an2* affects only

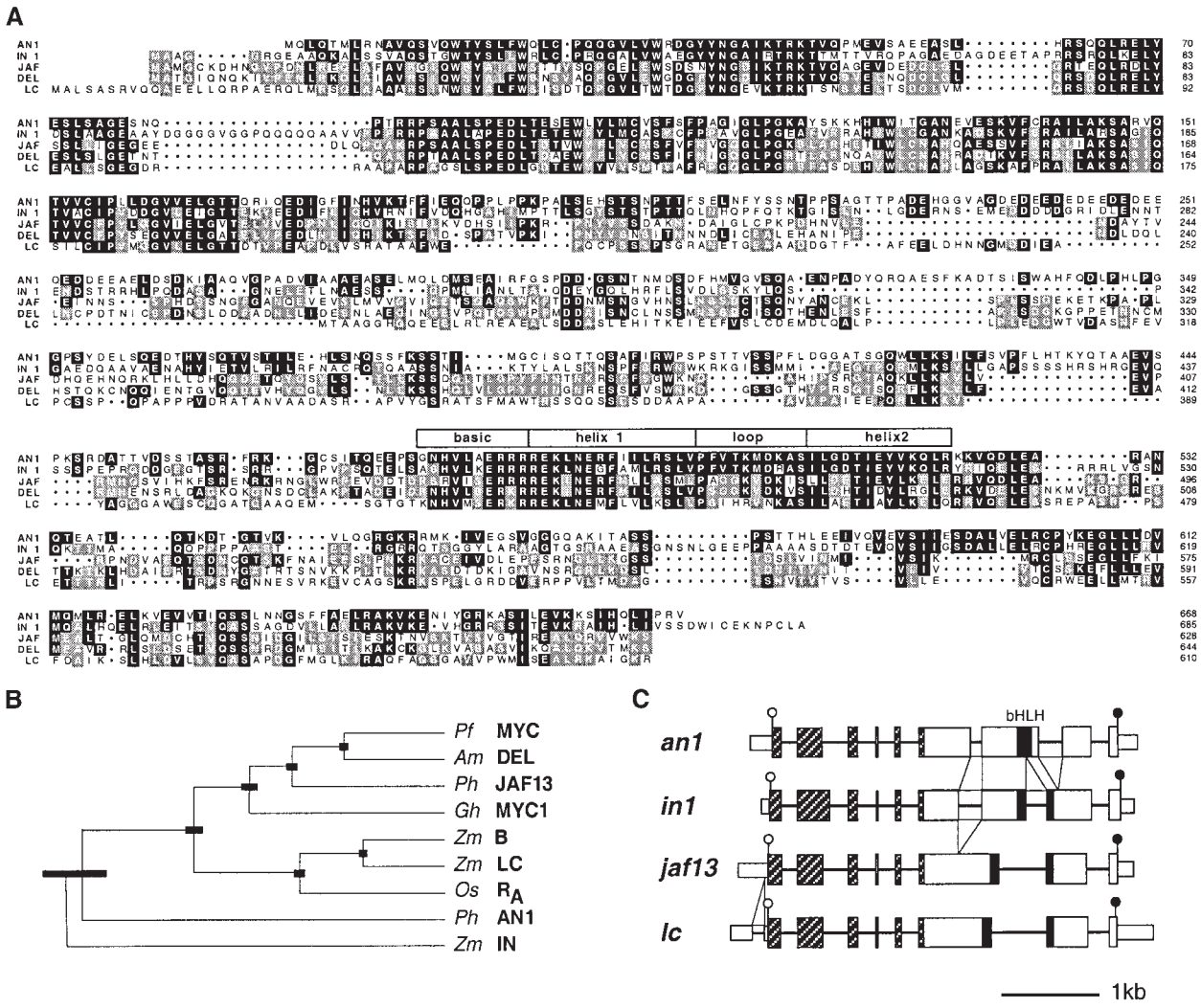

Figure 3. Molecular Analysis of the *an1* Gene.

(A) Alignment of the proteins encoded by *an1* and *jaf13* (JAF) from petunia, *in1* and *lc1* (LC) from maize, and *del* from snapdragon. Sequence identity between AN1 and any one of the other proteins is indicated by black shading. Identical amino acids that are not conserved in AN1 are indicated by gray shading. Numbering of the protein sequences is shown at right, and the position of the bHLH domain is indicated by the box overlying this region. Dots represent gaps introduced to improve the alignment.

(B) Phylogenetic tree of bHLH proteins implicated in pigmentation constructed by the unweighted pair method using arithmetic averages (UPGMA) algorithm (Sokal and Michener, 1958). The sequences are from the following sources: snapdragon (Am DEL, Goodrich et al., 1992; GenBank accession number M84913), *Perilla frutescens* (*Pf* MYC, Gong et al., 1999; accession number AB0204051), *Gerbera hybrida* (*Gh* MYC1, Elomaa et al., 1998; accession number AJ007709), rice (*Os* $R_{A,}$ Hu et al., 1996; accession number U39860), maize (*Zm* B, Radicella et al., 1991; accession number X57276; *Zm* LC, Ludwig et al., 1989; accession number M26227; *Zm* IN, Burr et al., 1996; accession number U57899), and petunia (*Ph* JAF13, Quattrocchio et al., 1998; accession number AF020543; and *Ph* AN1, this paper, accession numbers AF260918 and AF260919). The thick bars indicate the standard error in the positions of the branch point.

(C) Intron–exon structures of *an1*, *in1*, *jaf13*, and *lc1* (LC). Exons are drawn to scale, and coding sequences are indicated by double height. Introns are not drawn to scale but are presented in such a way that the conserved positions of 3′ acceptor splice sites are aligned. The positions of additional introns that are not conserved in all genes are indicated by the triangles between the diagrams. The regions encoding the conserved N-terminal domains and the bHLH domain are indicated by striped and black boxes, respectively. The open and closed circles denote the start and stop codons, respectively, of the protein coding region.

pigmentation in the petal limb. These results suggest that *an2* function is redundant with and taken over by other loci in distinct tissues (see Quattrocchio et al., 1998, 1999). In contrast, *an1* is expressed in all tissues that express *dfr* and *jaf13* (Figure 4). This, combined with the observation that mutation of *an1* blocks pigmentation in all tissues (de Vlaming et al., 1984; Quattrocchio et al., 1993), indicates that *an1* function is only barely, if at all, redundant.

Forced Expression of AN1 and AN2 Causes Ectopic *dfr* Transcription

To test whether AN1 is a transcription activator or a repressor (as IN is) and to study how it regulates the tissue-specific expression pattern of *dfr*, we expressed AN1 ectopically, either alone or in combination with AN2, and examined the activation of the *dfr* promoter. To this end, the *an1* cDNA containing the full 2004-bp open reading frame was fused to the cauliflower mosaic virus 35S promoter, 35S-*an1*. Similar constructs, 35S-*an2* and 35S-*jaf13*, were used to express AN2 and JAF13 (see Quattrocchio et al., 1998). Different combinations of these regulators were introduced into petunia leaf cells by particle bombardment; their activity was measured by monitoring the expression of a cobombarded *luciferase* reporter gene that was driven by the *dfrA* promoter, *dfr-luc*. Figure 5A shows that expression of either AN1 or JAF13 alone did not activate the *dfr* promoter, and expression of AN2 caused only weak induction. However, coexpression of AN2 plus AN1 or AN2 plus JAF13 strongly induced the *dfr* promoter, although coexpression of all three regulators did not further upregulate *dfr* promoter activity (data not shown).

In maize kernels, the *anthocyaninless1* gene (*a1*) encoding DFR is activated by C1 and R1 in the aleurone, whereas in the pericarp, the activator is P1, a MYB-type transcription factor. In maize suspension cells, forced expression of P1 alone is sufficient to induce transcription of *a1* (Grotewold et al., 1994; Bruce et al., 2000), and this induction cannot be enhanced by coexpression of R1; this indicates that P1, unlike C1, functions independent of R1-like bHLH factors (Grotewold, 1995; Sainz et al., 1997).

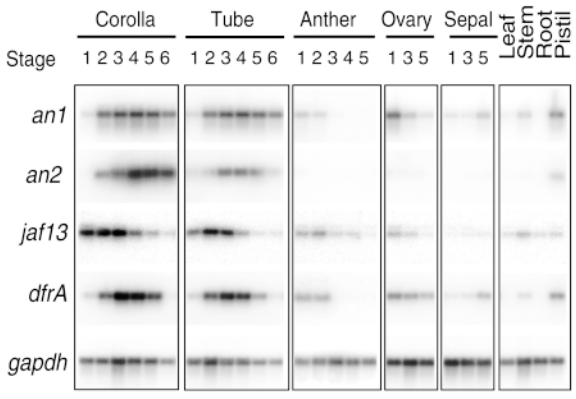

Figure 4. Spatiotemporal Expression Pattern of *an1*.

Transcripts of *an1*, *an2*, *jaf13*, *dfrA*, and *gapdh* were detected by quantitative RT-PCR in the corolla and tube, anthers, ovaries, and sepals from flowers at various developmental stages and from leaves, stems, roots, and pistils of the wild-type line V30, as indicated above the lanes.

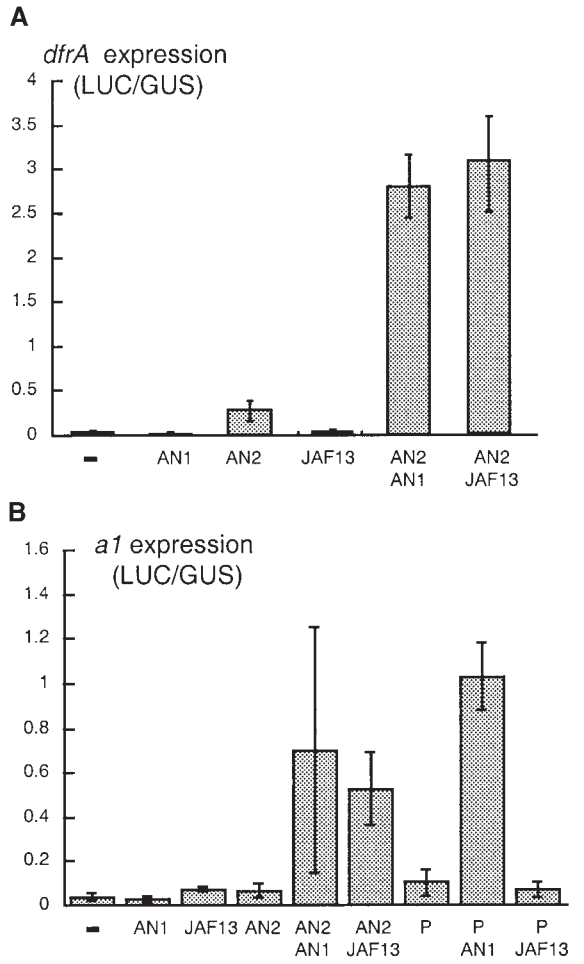

Figure 5. Activation of *dfr* and *a1* by Ectopically Expressed AN1, AN2, JAF13, and P.

(A) Activation of *dfr-luc* by AN1, AN2 and JAF13.
(B) Activation of *a1-luc* by AN1, AN2, JAF13 and P.
The columns and error bars in **(A)** and **(B)** denote the mean and standard error of the activity of the *dfr-luc* or *a1-luc* reporter gene after bombardment with 35S-*an1*, 35S-*an2*, 35S-*jaf13*, or 35S-*p* alone or in combination. Reporter gene activity, measured as luciferase (LUC) enzyme activity, is expressed in arbitrary units and was normalized to GUS enzyme activity expressed from a cobombarded reference gene, 35S-*gus*, which consisted of the β-glucuronidase gene driven by the 35S promoter.

Combined expression of AN2 and JAF13 could also activate the *a1* promoter in particle-bombarded petunia cells (Quattrocchio et al., 1998). Figure 5B shows that the combined expression of AN2 and AN1 or of AN2 and JAF13 activates the *a1* promoter with similar efficiency. In these petunia cells, the *a1* promoter could also be (weakly) activated by expression of P1 from a cobombarded 35S-*p1* transgene. Strikingly, the activation by P1 was enhanced ~10-fold by coexpression of AN1, whereas JAF13 had no effect (Figure 5B), indicating that P1 responds differently to AN1 and JAF13.

We conclude from these experiments that AN1 is a transcription-activating protein functionally different from JAF13 (and R1) and that it is an important determinant of the tissue-specific expression pattern of *dfr* and other structural anthocyanin genes.

AN1 Is Directly Involved in Transcription Activation of *dfr* and *myb27*

Previous experiments had shown that *an1*$^-$ petals lack transcripts of at least nine structural anthocyanin genes (see, e.g., Quattrocchio et al., 1993; Kroon et al., 1994; de Vetten et al., 1999) and of one potential regulatory gene, *Pmyb27*, which encodes a protein with similarity to MYB-domain transcription factors (Mur, 1995). Thus, AN1 might induce *dfr* transcription directly (e.g., as part of the transcription complex on the *dfr* promoter) or indirectly (by inducing the expression of an intermediate regulator such as MYB27). To distinguish between these possibilities, we constructed the 35S-*an1 gr* gene, in which the 35S promoter drives the expression of a chimeric protein consisting of the complete AN1 sequence and the ligand binding domain of the rat glucocorticoid receptor (GR), and introduced this gene into the *an1*$^-$ line W242. Treatment with dexamethasone (DEX), a synthetic steroid, was expected to induce AN1GR activity by releasing the protein from an inhibitory cytoplasmic complex rather than by stimulating de novo synthesis. In case protein synthesis were blocked, DEX treatment would still activate transcription of primary target genes but not of the secondary target genes controlled indirectly by AN1. In addition, we generated transgenic W242 plants containing a 35S-*an1* transgene or the empty vector.

We obtained 10 transformants harboring 35S-*an1*, three of which had full-colored flowers indistinguishable from those of the isogenic *An1*$^+$ line M87, indicating that the transgene fully complemented the *an1*$^-$ mutation. The other seven transformants had white, pale, or spotted flowers, apparently because of poor or irregular expression of the transgene. All 20 primary 35S-*an1 gr* transformants had white (*an1*$^-$) flowers similar to those of transgenic plants containing the empty vector. However, dipping single 35S-*an1 gr* flowers in a solution of DEX led in ~16 hr to full restoration of anthocyanin synthesis in six of the transgenics (Figure 6A). In the other 14 transformants, DEX treatment had little or no effect on pigmentation, presumably because of poor expression of the 35S-*an1 gr* transgene.

To study in further detail how AN1 induces pigmentation, we switched to a more easily controlled induction system in which detached flower buds were incubated with specific solutions in a test tube (Figure 6B); we monitored expression of the AN1 target genes by quantitative RT-PCR. Figure 6C shows that in buds containing 35S-*an1 gr*, the *dfr* and *myb27* mRNAs became detectable within 2 hr after DEX treatment and that after 20 hr, the quantities of these transcripts were comparable to those in fully colored buds harboring the constitutive 35S-*an1* transgene. No induction of *dfr* and *myb27* mRNA was seen in buds of transformants harboring the empty vector, confirming that DEX induction requires the AN1GR protein.

Pilot experiments showed that in the presence of 100 μM cycloheximide (CHX), DEX could no longer induce pigment synthesis, indicating that translation was effectively blocked (Figure 6B), whereas at 50 μM or less CHX, DEX-induced pigmentation was still detectable. RT-PCR analysis showed that the presence of 100 μM CHX did not reduce DEX-induced *dfr* and *myb27* mRNA amounts within the first 2 hr (Figure 6C, lane 18). Treatment with CHX alone had no clear effect on *dfrA* expression but did cause a weak induction of the *myb27* mRNA. After 20 hr of treatment with DEX and CHX, the *dfr* and *myb27* mRNAs decreased to barely detectable amounts in 35S-*an1 gr* flowers (lane 20) but remained high in 35S-*an1* flowers (lane 12). We believe that this difference is due to the relatively low stability of the AN1GR fusion protein compared with AN1, combined with the continuous turnover of *dfrA* and *myb27* transcripts during the course of the experiment.

Because it was crucial to assess whether translation was completely blocked under the experimental conditions used, we analyzed the expression of a β-*glucuronidase* (*gus*) reporter gene driven by the *dfr* promoter. We backcrossed an *An1*$^+$ dfr-*gus* transgenic plant (Huits et al., 1994a) with a 35S-*an1 gr* plant (as the recurrent parent) and selected *an1*$^-$ 35S-*an1 gr dfr-gus* plants from the progeny. Flower buds from these plants were analyzed for induction of *dfr* and *gus* expression, as described above. Figure 6D shows that DEX induced *dfr* and *gus* mRNA to a similar extent, irrespective of whether CHX was present or not. However, in the presence of CHX, no appreciable GUS enzyme activity was found, confirming that translation was completely blocked.

We conclude from these results that AN1 is a direct activator of *dfrA* transcription and does not operate by inducing the transcription or translation of a hitherto unknown intermediary regulator.

Regulation of *an1* Expression by *an2* and *an4*

Because *an1* functions relatively late in the regulatory hierarchy, the question arose whether *an2*, *an4*, or *an11* also controls pigmentation directly or by regulating *an1* expression.

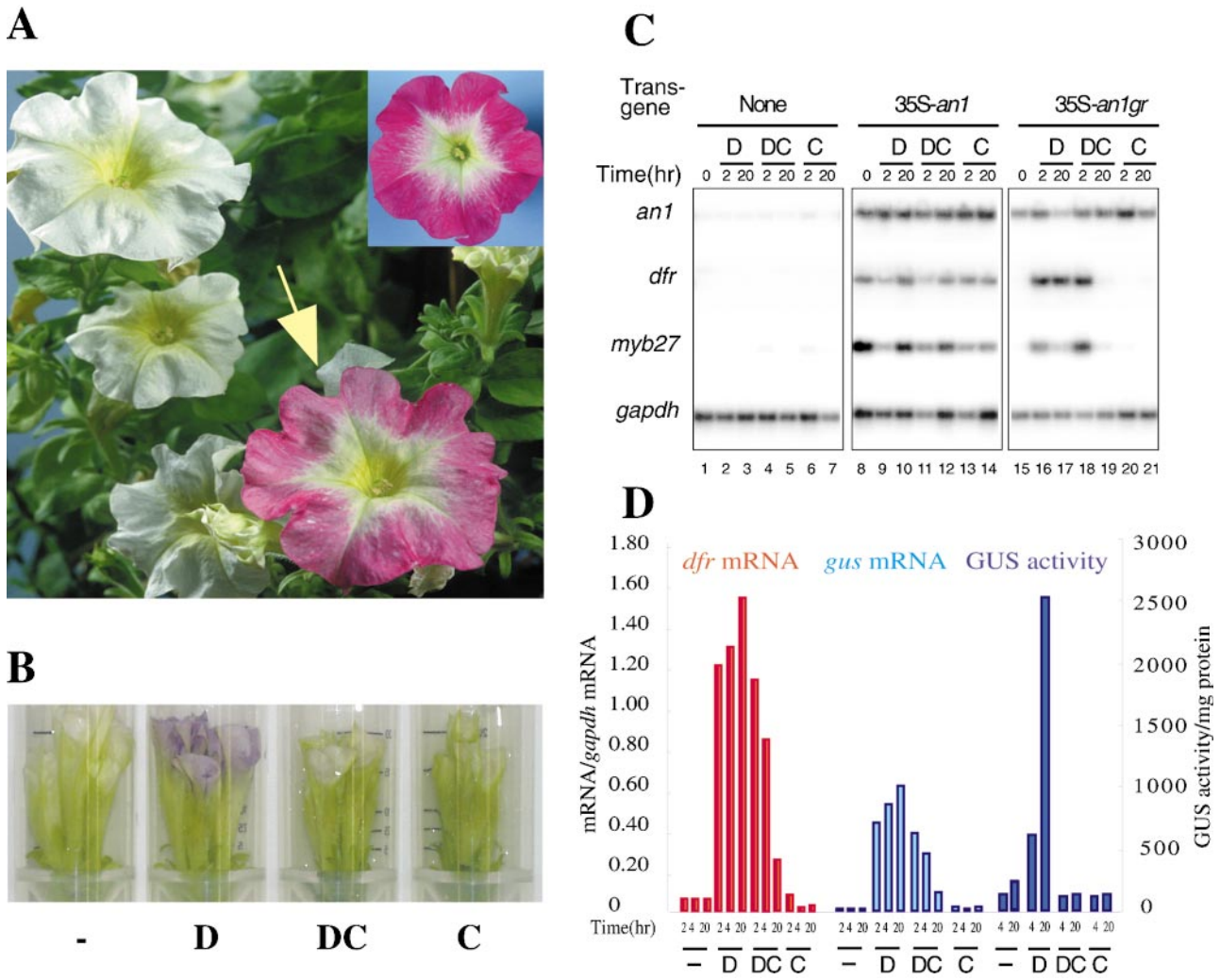

Figure 6. Direct Activation of *dfr* and *myb27* by an AN1GR Fusion Protein.

(A) A plant of the *an1*$^{-}$ line W242 harboring 35S-*an1*, 20 hr after one floral bud (arrow) had been dipped in 10 μM DEX. For comparison, the inset in the top right corner shows a flower of a transgenic W242 plant complemented with the 35S-*an1* transgene.

(B) Phenotype of a transgenic W242 flower harboring 35S-*an1 gr* after 20 hr of incubation in a test tube containing water (−), 10 μM DEX (D), 10 μM DEX plus 100 μM CHX (DC), or 100 μM CHX (C).

(C) Detection of *an1*, *dfr*, *myb27*, and *gapdh* transcripts in petal limbs of W242 flowers containing 35S-*an1 gr*, 35S-*an1*, or the empty vector after incubation in 10 μM DEX (D), 10 μM DEX plus 100 μM CHX (DC), or 100 μM CHX (C) for 2 or 20 hr. The numbering of the lanes is indicated at the bottom.

(D) *dfr-GUS* expression in *an1*$^{-}$ mutant plants harboring 35S-*an1 gr*. Flowers treated for various periods with DEX or CHX or both, as indicated in **(B)** and **(C)**, were assayed for *gus* mRNA, *dfr* mRNA, and GUS enzyme activity. *gus* and *dfr* mRNA amounts, expressed in arbitrary units, were determined by hybridization of RT-PCR products, quantification of the radioactivity by PhosphorImaging, and normalization to the amount of *gapdh* mRNA.

To address this question, we examined by RT-PCR the expression of *an1*, *an2*, *jaf13*, and *an11* mRNA in various mutants.

The *an11* gene is, like *an1*, required for pigmentation of all tissues, including the petal limb (de Vlaming et al., 1984; Quattrocchio et al., 1993). RT-PCR analysis showed that petal limbs of the isogenic lines R27 (*An11*$^{+}$) and W134 (*an11*$^{-}$) contained similar amounts of *an1* transcripts (data not shown). Because *an11-W134* homozygotes lack detectable AN11 protein (de Vetten et al., 1997), this suggests that AN11 is not required for *an1* transcription (data not shown).

RT-PCR analysis showed that petal limbs from line W82, which harbors the unstable *dTph1* insertion allele *an2-W82*

(Quattrocchio et al., 1999), and those from derived *An2*$^+$ germinal revertants contained similar amounts of *an1* transcripts (data not shown). However, because *an2-W82* is not a null allele, this result is difficult to interpret. Therefore, we analyzed *an1* transcripts in an F_1 hybrid of the lines W115 and W59, both of which contain frameshift *an2* alleles that have completely lost the ability to activate *dfrA* (Quattrocchio et al., 1999), and compared them with *an1* mRNA in complemented W115/W59 plants harboring a 35S-*an2* transgene. Figure 7 shows that the 35S-*an2* complementants express high amounts of *an2* mRNA, which restores *dfr* expression (lanes 4 to 6) and pigmentation (see Quattrocchio et al., 1998). However, complementation does not affect the amount of *an1* (or *an11* or *jaf13*) mRNA, suggesting that *an2* is not essential for *an1* expression in the petal limb.

Mutation of *an4* blocks pigmentation of the anthers but not of the corolla limb. Initial experiments showed that anthers of line R27 (*an4*$^-$) lacked *an1* transcripts (data not shown), whereas the transcripts were detectable in anthers of line V30 (*An4*$^+$) (Figure 4). To "normalize" for differences in the R27 and V30 genetic backgrounds, we analyzed anthers pooled from multiple An1$^+$An4$^+$ and An1$^+$an4$^-$ plants obtained by the backcross (W138 × V30) × W138. Figure 7 shows that An4$^+$ anthers express *dfr* and *an1* mRNA, as expected (lanes 10 to 12), whereas both transcripts are downregulated in *an4*$^-$ anthers (lanes 7 to 9). The *an4*$^-$ genotype did not, however, affect expression of *an11*, *jaf13*, or *gapdh* mRNA.

Previous experiments showed that the introduction of a 35S-*an2* gene in the *an4*$^-$ line W115 and the hybrid W115/W59 restored pigmentation in the corolla (complementation of *an2*) as well as the anthers, indicating that ectopically expressed AN2 complements *an4* (Quattrocchio et al., 1998). Figure 7 shows that *an4*$^-$ anthers of W115/W59 contain very small amounts of *dfr* and *an1* mRNA (lanes 13 to 15). As expected, *dfr* mRNA quantities are upregulated again in anthers of the 35S-*an2* complementants, although the amount of *dfrA* mRNA remains less than that in the An4$^+$ V30/W138 plants, possibly because of differences in the genetic background. Surprisingly, the amounts of *an1* mRNA in these complemented anthers were restored to the same extent as was *dfr* (lanes 16 to 18). Moreover, whereas *dfrA*, *an1*, and *jaf13* mRNAs disappear simultaneously in An4$^+$ anthers when ageing, the expression of *dfrA* and *an1* is prolonged relative to *jaf13* in *an4*$^-$ 35S-*an2* anthers, apparently because 35S-*an2* expression persists during anther development (lanes 16 to 18).

In leaves of transgenic plants, ectopic expression of AN2 activated the expression of *dfrA*, but not of the early structural genes *chsA*, *chi*, and *f3h*, and increased pigmentation in the veins (Quattrocchio et al., 1998). Figure 7 shows that AN2 also induces the expression of *an1* mRNA in this tissue, up to amounts ordinarily found in floral tissues only, whereas AN2 has little or no effect on the expression of *jaf13* and *an11*.

Together, these data indicate that expression of *an1* is controlled by *an2* and *an4* (see also Discussion).

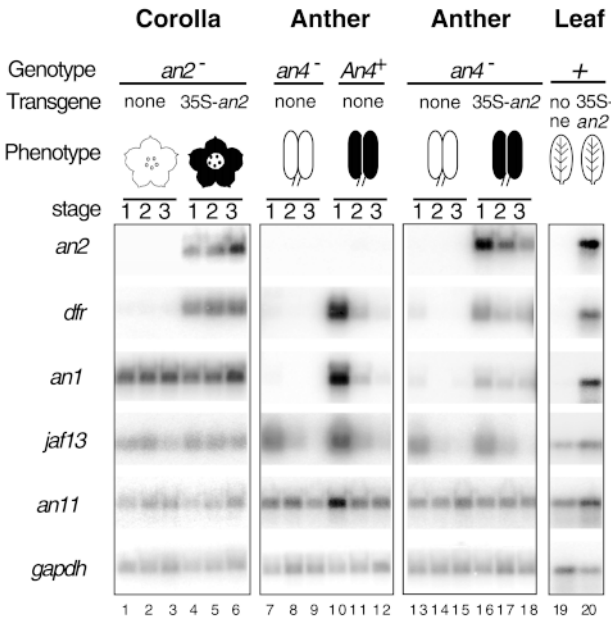

Figure 7. Genetic Control of *an1* Expression.

Transcripts of *an2*, *dfr*, *an1*, *jaf13*, *an11*, and *gapdh* were detected by RT-PCR in different developmental stages of *an2*$^-$ corollas from the hybrid W115/W59 and complementants harboring 35S-*an2* (lanes 1 to 6), in anthers from selected *an4*$^-$ and *An4*$^+$ progeny of the backcross (W138 × V30) × W138 (lanes 7 to 12), in *an4*$^-$ anthers from the hybrid W115/W59 and complementants harboring 35S-*an2* (lanes 13 to 18), and in leaves of W115/W59 and W115/W59 plants harboring 35S-*an2* (lanes 19 and 20). The presence of anthocyanin pigments in the various tissues is indicated by cartoons. The numbering of the lanes is indicated at bottom.

DISCUSSION

AN1 Represents a bHLH Protein That Is Distinct from JAF13, DEL, and R1

The AN1 protein has high similarity with R1 from maize and DEL from snapdragon, particularly in the ~170–amino acid N-terminal domain and in the bHLH domain. Given the similar phenotypes of *an1*$^-$, *r1*$^-$, and *del*$^-$ mutants, the question arises whether *an1*, *r1*, and *del* are orthologous genes. If so, this may imply that *jaf13*, previously identified by homology to *r1* and *del* and by gain-of-function studies, is another member of the same gene family, possibly with little or no activity. However, numerous findings indicate that this idea is incorrect; rather, they suggest that *an1* is a novel regulator and cannot be considered the ortholog of *r1* and *del*. For example, if the many insertions and deletions are ignored, AN1 is more similar to IN than to R1 or DEL (Figure 3). Moreover, R1 is more similar to petunia JAF13 than it is to AN1, and the intron–exon structure of the *r1* gene is more similar to

that of *jaf13* than that of *an1*. Functional assays also reveal differences between AN1 and JAF13 or R1. First, AN2 can induce expression of *an1* but not of *jaf13* (Figure 7). Second, activity of the P1 protein can be enhanced by AN1 but not by JAF13 (Figure 5B) or R1 (Grotewold, 1995; Sainz et al., 1997). Third, using two-hybrid screens in yeast, we identified a protein that interacts with the bHLH domain of AN1 but fails to recognize JAF13 in yeast (A. Kroon and R. Koes, unpublished results).

Collectively, these findings argue strongly against *an1* being the petunia ortholog of *r1* and *del*; they are better explained by assuming that the common ancestor of monocot and dicot plants had two genes coding for bHLH proteins, one of which is the ancestor of today's *r1*, *delila*, and *jaf13* and the other of *an1*.

The interaction between P1 and AN1 in activating the *a1* expression in petunia cells may seem surprising, given that P1 controls the synthesis of phlobaphenes, a type of flavonoid pigment not present in petunia flowers. However, the pigment responsible for the brown color of petunia seeds is unknown, although it can be converted into an anthocyanin (delphinidin) by treatment with mineral acid. That its synthesis requires some of the flower pigmentation loci, including *an1*, *an11*, and *an6* (*an6* contains the *dfrA* gene) (Koes et al., 1990), suggests that it may a be flavonoid polymer such as, for instance, condensed tannins or a phlobaphene. Thus, the interaction of AN1 with P1 may reflect interaction with an unknown MYB protein from petunia involved in pigmentation of the seed coat.

Regulation of *an1* Expression

In maize, transcription of *c1* is controlled by VIVIPAROUS1 (VP1), a transcriptional regulator that controls multiple aspects of seed maturation, including anthocyanin synthesis, seed dormancy, and expression of amylase genes (Hoecker et al., 1995; Suzuki et al., 1997). Our data on *an1* indicate that the anthocyanin-specific regulators also operate in some sort of transcriptional hierarchy.

AN4 is required for expression of *an1* in anthers (Figure 7). Several observations indicate that *an4* encodes a paralog of AN2 or controls its expression. *an2* and *an4* mutants have complementary phenotypes; they result in loss of pigmentation in either the petal limb (*an2*$^-$) or the anthers (*an4*$^-$) with little or no effect on pigmentation of the seed coat or the flower stem (de Vlaming et al., 1984; Quattrocchio et al., 1993). Moreover, *an4* mutants are complemented by ectopic expression of AN2, as judged by the restoration of pigmentation (Quattrocchio et al., 1998) and expression of *dfrA* and *an1* (Figure 7). Finally, we recently identified two new MYB-type proteins that appear, based on sequence homology and expression patterns, to be paralogs of AN2. One of these genes is strongly expressed in *An4*$^+$ anthers but not in *an4*$^-$ anthers (A. Kroon, C. Spelt, and R. Koes, unpublished data).

In gain-of-function experiments, AN2 (re)activates *an1* expression in *an4*$^-$ anthers (complementation of *an4*) and in leaves, a tissue that normally does not express the anthocyanin pathway. This effect of AN2 appears to be specific for *an1*, because structural anthocyanin genes such as *chs*, *chi*, and *an3*, which are regulated independent of *an1*, *an2*, *an4* and *an11*, are not activated in 35S-*an2* leaves (Quattrocchio et al., 1998); moreover, AN2 does not have a clear effect on the expression of *jaf13* or *an11* (Figure 7).

The conclusion that like *an4*, *an2* is a regulator of *an1* expression seems inconsistent with the finding that *an1* transcripts are still expressed in *an2*$^-$ petal limbs. This discrepancy can be explained in at least two ways. First, the *an2*$^-$ alleles of W115 and W59 express roughly wild-type amounts of mRNA and encode a protein that is truncated immediately after the MYB domain (Quattrocchio et al., 1999). Although these *an2*$^-$ alleles fail to activate *dfrA* transcription, even when expressed from the strong 35S promoter (Quattrocchio et al., 1999), the truncated AN2 protein might still be stable and capable of activating *an1* expression. Second, the function of *an2* seems redundant, even in the petal limb, because *an2*$^-$ petal limbs are pale rather than completely white and continue to express small amounts of structural anthocyanin mRNAs. The residual transcription activity presumably is provided by one of the above-mentioned AN2 paralogs, which may be sufficient for full activation of *an1* and only partial activation of the structural anthocyanin genes (which are expressed much more strongly than *an1*).

Despite several attempts, we have not been able to solve this apparent discrepancy. Experiments to test whether AN2 induces *dfrA* transcription indirectly (by way of *an1*) through expressing a DEX-regulated AN2GR protein in transgenic plants were unsuccessful because the AN2GR fusion protein failed to complement *an2* and *an4* mutations (apparently AN2GR is unstable or nonfunctional). Also, transient assays designed to test whether a truncated AN2 protein could activate *an1* transcription failed because a *luc* reporter gene fused to an 1.5-kb fragment upstream from the *an1* was transcriptionally inactive. Whether this is because important *cis*-acting regulatory elements of *an1* are located outside the promoter region or because this region of *an1* represents a large intron rather than the promoter is unclear at this stage.

Activation of Structural Anthocyanin Genes

Although the first regulatory anthocyanin genes, *c1* and *r1*, were isolated >10 years ago, we still know very little about the mechanism by which they activate the structural anthocyanin genes. In vitro DNA binding studies showed that C1 can bind to functional *cis*-acting elements in the promoters of the *a1* and *a2* genes (Sainz et al., 1997; Lesnick and Chandler, 1998). However, because C1 binds with relatively low affinity to *a1* (Sainz et al., 1997) and because multiple MYB-domain proteins may be involved in the activation of structural anthocyanin genes (see Solano et al., 1995; Quattrocchio et al., 1999), it is difficult to judge whether the DNA–protein interactions observed in vitro are relevant in

vivo. For R1, neither direct DNA binding nor an enhancement of the DNA binding capacity of C1 could be demonstrated, even though R1 is known to be required for transcriptional activation by C1. Because nearly all available data are from genetic experiments, it is unclear whether the identified regulators activate the structural anthocyanin genes directly or are part of a more complex regulatory hierarchy in which they regulate the structural genes in an indirect manner. Obviously, such information is important for the correct interpretation of the in vitro analyses of DNA–protein interactions.

Our results show that AN1 is both an activator of transcription (not an inhibitor as IN is) and a key factor in determining the expression domain of *dfr* (Figure 5). In transient assays, expression of AN1 together with AN2 is sufficient to activate the *dfrA* promoter in leaf cells. Surprisingly, the function of AN1 can be fully replaced by JAF13 in such assays, even though the phenotype of *an1*$^-$ petals—which still normally express *jaf13* mRNA (Quattrocchio et al., 1998)—indicates that ordinarily JAF13 cannot replace AN1. Most likely, AN1 and JAF13 have different affinities for partner proteins or target DNA sequences, or both—subtle differences that are not recognizable when these proteins are produced in excess from a transgene driven by the strong 35S promoter.

In transient assays, expression of AN2 causes only weak activation of *dfrA-luc* or *dfrA-gus* reporter genes, and coexpression of a bHLH protein such as JAF13, AN1, or R1 is required for full activation of *dfrA* (Figure 4; Quattrocchio et al., 1998). In stable transformants, however, ectopic expression of AN2 alone is sufficient for a strong induction of *dfr* mRNA in leaves, up to amounts that ordinarily are seen in pigmented floral tissues only (Figure 7), and the effect of AN2 is enhanced only slightly by coexpression of JAF13 (Quattrocchio et al., 1998). These seemingly conflicting results are probably attributable to the different time spans of the experiments. In stable transformants, ectopic expression of AN2 induces the expression of AN1 (Figure 7) and possibly of other regulatory proteins required for subsequent activation of *dfr*. In transient assays, a period of only 20 hr elapses between the introduction of DNAs and the assay of reporter gene activity, which may be too little time for AN2 to induce expression of sufficient AN1 for activation of *dfr*. However, if 35S-*an1* is cointroduced, AN1 starts to accumulate immediately, resulting in a stronger expression of the *dfr* reporter gene during the 20 hr of the experiment.

The experiments in which the localization and activity of an AN1GR fusion protein could be controlled post-translationally strongly indicate that AN1 is a direct regulator of *dfr* and does not operate by activating the transcription/translation of an intermediate regulatory gene (Figure 7). The same strategy was used to demonstrate the direct regulation of *apetala1* by LEAFY and of *nap* (*nam-homolog activated by apetala3*) by APETALA3 in floral meristems of Arabidopsis (Sablowski and Meyerowitz, 1998; Wagner et al., 1999). However, our results do not necessarily imply that AN1 contacts the *dfr* promoter directly. AN1 may well be recruited to the transcription complex by protein–protein interactions only (as an adapter or coactivator), particularly because the putative DNA binding "basic" region upstream of the HLH domain in AN1 (as well as in JAF13, R1, DEL, and homologous genes from other plants) contains only a few basic amino acids in comparison with mammalian bHLH proteins with a proven DNA binding capacity.

METHODS

Petunia Lines and Mutants

The petunia lines R27 (*an4*$^-$), W138 (*an1-W138*, *an4*$^-$), and W137 (*an1-W137*, *an4*$^-$) are all from the same genetic background (R27). The line W242 contains a null allele of *an1*, which was obtained by the insertion and excision of a *dTph1* element in progeny of the *An1*$^+$ line M87 (C. Spelt, F. Quattrocchio, J.N.M. Mol, and R. Koes, unpublished data). Details on line W82 harboring the unstable *an2-W82* allele, the derived *An2*$^+$ revertants, and the *an2*$^-$*an4*$^-$ lines W115 and W59 have been described by Quattrocchio et al. (1999). Lines V30, V23, M87 (all wild type for all *an* genes), W82, W115, W59, and R27 are all from different genetic backgrounds. To obtain An4$^+$ and an4$^-$ anther tissue in a normalized genetic background, we used the backcross (W138 × V30) × W138. B_1 plants that were An1$^+$ (scored from coloration of the petals) and Hf1$^+$ (determined by thin-layer chromatographic analysis of petal anthocyanins) were selected, and anthers from multiple An4$^+$ (blue/purple anthers) or an4$^-$ (yellow anthers) plants were pooled for RNA isolation. All plants were grown in a normal greenhouse.

Molecular Analysis of the *an1* Locus

DNA prepared from an *an1-W138* homozygous plant was digested with RsaI, diluted, and circularized with T4 ligase. *dTph1* flanking sequences were amplified by inverse polymerase chain reaction (PCR), using *dTph1* specific primers and cloned into M13 phage to obtain a library of *dTph1* flanking sequences (Souer et al., 1995). Duplicate plaque lifts, taken on Hybond-N membranes (Amersham), were hybridized to ^{32}P-labeled *dTph1* flanking sequences generated by inverse PCR amplification of circularized RsaI-digested DNA from a second *an1-W138* homozygote (+ probe) or a plant homozygous for an *An1*$^+$ revertant allele (− probe). Two hundred differentially hybridizing plaques were isolated and grouped in ~20 classes by cross-hybridization and sizing of the insert. Representative clones of each class were hybridized to dot blots containing inverse PCR–amplified *dTph1* flanking sequences from four *an1-W138* unstable plants and from plants homozygous for four independent *An1*$^+$ revertant alleles. Clone 65 produced the hybridization pattern expected for an *an1* fragment, and further DNA gel blot and PCR analyses confirmed that it contained part of the *an1* locus (Figure 2).

Genomic and cDNA clones representing the *an1* locus and mRNA were isolated by hybridization of clone 65 to a cDNA library prepared from petal RNA and a genomic library of an *An1*$^+$ line (R27). Nested deletions of cDNA and genomic clones were sequenced by the dideoxychain termination method, using fluorescent primers complementary to the cloning vector and an Applied Biosystems (Foster

City, CA) DNA sequencer (model 370A). Sequences were analyzed with the program Geneworks (Intelligenetics, Mountain View, CA), and alignments were optimized by hand.

Construction of Transgenes and Plant Transformation

The 35S-*an1* gene was constructed by ligation of the 2.45-kb *an1* cDNA between the 35S promoter of cauliflower mosaic virus and the 3′ end of the *nopaline synthase* gene of plasmid PIP M1. For construction of 35S-*an1 gr*, primers 1.20 and 1.34 were used to amplify the complete coding region (without stop codon) of *an1*; this fragment was inserted into PIP M1, together with a fragment encoding the ligand binding domain (amino acids 508 to 795) of the glucocorticoid receptor (GR) obtained from the plasmid pBI-ΔGR (Lloyd et al., 1994). Details of the genes 35S-*jaf13*, 35S-*an2*, *dfrA-gus*, *dfrA-luc*, and 35S-*P1* can be found elsewhere (Grotewold et al., 1994; Huits et al., 1994a; de Vetten et al., 1997; Quattrocchio et al., 1998).

For plant transformation, 35S-*an1* and 35S-*an1 gr* were inserted into the binary T-DNA vector pBIV M2 and introduced into the *an1*$^-$ line W242 by leaf-disc transformation. Transgenic plants containing 35S-*an2* were generated by Agrobacterium-mediated leaf-disc transformation of F_1 plants from the cross W115 × W59.

Transient transformation of petunia leaf cells was performed by particle bombardment, as described previously (de Vetten et al., 1997; Quattrocchio et al., 1998).

Dexamethasone Induction Experiments

Transgenic plants expressing the AN1GR fusion protein were selected by dipping stage 4 floral buds in 10 μM dexamethasone (DEX) and visually inspecting for pigment synthesis after ~16 to 20 hr, when the flower had opened.

For treatments with DEX or cycloheximide (CHX), or both, stage 4 floral buds were taken from the plants and incubated in transparent 50-mL test tubes with 10 mL of water containing 10 μM DEX or 100 μM CHX (or both) on a rocking and rolling test tube mixer. This ensured that the tissues were continuously exposed to the solution without being completely submerged.

Expression Analyses

RNA isolation, RNA gel blot analyses, and reverse transcription (RT)–PCR were performed as described previously (Quattrocchio et al., 1993; de Vetten et al., 1997). In comparing mRNA quantities in different genotypes, care was taken that the analyzed tissues were harvested simultaneously. Radioactive hybridization products were detected and quantified by using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Transient expression assays were performed by particle bombardment of petunia W115 leaves, as described previously (de Vetten et al., 1997; Quattrocchio et al., 1998)

ACKNOWLEDGMENTS

We thank Jan Kooter for commenting on the manuscript, Erich Grotewold for a gift of the 35S-*p1* construct, Alan Lloyd for a gift of pBI-ΔGR, and Daisy Kloos, Pieter Hoogeveen, and Martina Meesters for their care of the plants. This research was sponsored in part by the Netherlands Organisation for Scientific Research and by a grant from the European Union (No. BIO4-CT98-0432).

Received April 26, 2000; accepted July 3, 2000.

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